ACTIVATION OF THE PYRUVATE DEHYDROGENASE COMPLEX IN ISOLATED FAT CELL MITOCHONDRIA BY HYDROGEN PEROXIDE AND *t*-BUTYL HYDROPEROXIDE

I. PAETZKE-BRUNNER and O. H. WIELAND
with the technical assistance of G. FEIL

Forschergruppe Diabetes und Klinisch-chemisches Institut, Städtisches Krankenhaus München-Schwabing, Kölner Platz 1, 8000 München 40, FRG

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1. Introduction

Hydrogen peroxide (H_2O_2) has been reported to mimic several effects of insulin in rat adipocytes such as stimulation of glucose transport [1], of glucose C-1 oxidation [2], of glucose incorporation into glycogen [3], of lipid synthesis from glucose [4], and of pyruvate dehydrogenase complex (PDC) activity [4,5]. Evidence has also been presented that insulin leads to an increase in H_2O_2 production in adipocytes [6,7] due to activation of a NADPH oxidase localized in the plasma membrane [8]. From these findings the possibility was raised that H_2O_2 might play a role as second messenger of insulin in adipose tissue [4,5].

As the PDH complex is located inside the mitochondria it seemed of particular interest to examine if H_2O_2 might be capable to activate the enzyme when added directly to isolated mitochondria. As will be shown here isolated fat cell mitochondria displayed much greater responsiveness of PDC to H_2O_2 in comparison to isolated fat cells. Moreover we could demonstrate that an organic peroxide, *t*-butyl hydroperoxide is even superior to H_2O_2 in stimulating PDC activity in isolated mitochondria. As judged from measurements of mitochondrial ATP levels the peroxides at the low concentrations used did not interfere with mitochondrial energy production. From our results it thus appears that isolated fat cell mitochondria represent a preferred object for further studies into the mechanism(s) of PDC activation by peroxide(s).

2. Materials and methods

Enzymes, coenzymes were from Boehringer (Mannheim), bovine serum albumin (BSA), dried, purified from Behring Werke AG, (Marburg-Lahn), collagenase CLS from Worthington (Freehold NJ), *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (Hepes), morpholinopropane sulfonic acid (MOPS) and β-phenylethylamine from Serva (Heidelberg), Lubrol PX from ICI (Frankfurt), silicon oil AK 20 from Wacker Chemie (Burghausen). Other chemicals were from Merck (Darmstadt). Sodium [^{14}C]formate (spec. act. 4.4 Ci/mol) was purchased from New England Nuclear and pig insulin was a gift of Novo (Mainz).

ATP and ADP were determined by enzymatic methods with an Aminco-Bowman spectrophotofluorometer (Am. Inst. Co.) as in [9]. H_2O_2 production was measured by the oxidation of sodium [^{14}C]-formate, according to [7] with the exception that formate was 0.2 mM. The reaction was stopped with 0.5 ml 1.7 M CH_3COOH, and $^{14}CO_2$ was absorbed on filter strips presoaked in 0.4 ml 25% phenylethylamine in methanol. The whole procedure resembles in principle that described for measurement of [^{14}C]-glucose oxidation [10]. Isolated fat cells were prepared from epididymal adipose tissue of normal fed Sprague-Dawley rats (Ivanovas, Kisslegg) weighing 160–200 g by the collagenase method [11] using 2 ml/g fat pad of 'Hepes-buffer': 131 mM NaCl; 4.8 mM KCl; 2.5 mM $CaCl_2$; 1.2 mM KH_2PO_4; 1.2 mM $MgSO_4 \times 2$ H_2O; 25 mM Hepes; 50 mg BSA/ml; and 210 U collagenase/ml; final pH was 7.4. Digitonin fractionation of fat cells [12] and preparation of fat cell mitochondria [13] was done as described.

2.1. *Incubation of intact fat cells*

Packed cells (0.2 ml) were incubated with gentle

shaking in closed plastic flasks at 37°C in 1.8 ml 'Hepes-buffer' containing 25 mg BSA/ml. For other additions see individual experiments.

2.2. *Incubation of mitochondria*

As a rule 50 μl mitochondrial suspension were incubated at 25°C in Eppendorf cups after mixing with 1.45 ml KCl-medium (130 mM KCl, 2 mM $MgCl_2$, 2 mM EDTA, 2 mM KH_2PO_4, 0.05% BSA, 5 mM Tris–HCl (pH 7.4)). Gas phase was air. At the end of incubation the samples were centrifuged for 60 s at 9000 × *g* and 5°C (Eppendorf centrifuge), and the supernatant discarded. Samples for enzyme determination were frozen in liquid nitrogen and further treated as described below. For adenine nucleotide determination the pellets were immediately deproteinized by vigorous mixing (Whirl-Mix) with 0.1 ml 12% $HClO_4$ and further treated by neutralization with KOH and centrifugation as usual. As a rule the contents of 3 cups were pooled for adenine nucleotide determination.

2.3. *Preparation of enzyme extracts*

Extracts from whole fat cells were prepared as in [12]. The frozen mitochondrial pellets were suspended by vigorous agitation (Whirl-Mix) in 0.2 ml 15 mM Tris–HCl (pH 7.0) containing 0.01 ml rat serum [14] and 0.002 ml Lubrol PX. After another freezing and thawing of the samples PDC activity was measured spectrophotometrically before (PDH_a), and after incubation of the extract with pig heart phosphatase [15] and 10 mM Mg^{2+} as in [13] (total activity).

3. Results and discussion

Incubation of adipocytes in the presence of 5 mM H_2O_2 caused a nearly 3-fold increase in the active form of pyruvate dehydrogenase (table 1). The possibility that this change could be due to a fall in mitochondrial ATP caused by H_2O_2 appears to have not been considered previously. It seems therefore important to note from table 1 that in these experiments, like in earlier studies with insulin [12] the ATP levels remained essentially unchanged when measured in the mitochondrial compartment of these cells obtained by digitonin fractionation.

With regard to the possible role of H_2O_2 acting as an intracellular messenger of insulin at the mitochondrial level it was of interest to investigate if hydrogen peroxide has an effect on the PDH complex also when directly added to incubations of isolated mitochondria. If one compares the results of table 1 and table 2 it is clear that isolated mitochondria respond to much lower concentrations of H_2O_2 than whole cells showing a 3-fold increase in the amount of PDH_a at 0.1 mM H_2O_2. It should be noted that again there was no lack in ATP supply as indicated by the unchanged ATP/ADP ratio under these conditions whereas a marked fall of mitochondrial ATP/ADP did occur at higher H_2O_2 concentrations (1 mM) which apparently lead to a disturbance of the mitochondrial energy metabolism. The large additional increase of

Table 1
Effect of H_2O_2 on PDH_a activity and ATP-levels of isolated fat cells and their mitochondrial fraction

Additions	PDH_a (in % of total)	ATP (nmol/g fresh cells)	
	Whole cells	Whole cells	Mitochondrial fraction
None	13.4 ± 3.1 $p < 0.005$	120.6 ± 9.7 n.s.	7.5 ± 1.0 n.s.
H_2O_2 (5 mM)	29.6 ± 4.0	113.2 ± 1.8	6.9 ± 0.9

Cells were incubated 15 min in glucose (0.5 mM) containing medium and further treated for digitonin fractionation. Total PDC activity was 289 ± 29 mU/g fresh cells. Mean values ± SEM of 4 different expt. assayed in triplicate are given. For further details see section 2. Statistical significance against control without H_2O_2 was measured by Student's *t*-test.

Table 2
Effect of H_2O_2 on PDH_a activity and ATP/ADP ratio of isolated fat cell mitochondria

Addition	PDH_a (in % of total)	$\frac{ATP}{ADP}$
None	10.5 ± 2.0	0.54 ± 0.06
H_2O_2, 0.1 mM	33.1 ± 5.6[a]	0.53 ± 0.07
H_2O_2, 1.0 mM	70.3 ± 7.8[a]	0.31 ± 0.04[a]
ATP-trap	72.1 ± 5.4[a]	0.25 ± 0.04[a]

[a] $p < 0.01$

Fat cell mitochondria corresponding to a total PDC activity of 18.4 ± 4.2 mU were incubated in 1.5 ml KCl-medium containing 0.05% BSA and where indicated 20 μM ADP, 10 mM glucose, 250 μg/ml hexokinase for ATP trapping. Incubation time was 9 min. *t* = 25°C. For further details see section 2. Values are given as means ± SEM of 5 expt.

the level of PDH_a up to 70% of total PDC activity at high doses of H_2O_2, an effect comparable to that obtained by ATP trapping in table 2 must therefore clearly be distinguished from the mitochondrial actions of low peroxide concentrations. Thus, all subsequent studies were carried out using peroxide concentrations which did not influence the phosphorylation state of the mitochondrial adenine nucleotides.

The time course of PDC activation by H_2O_2 in isolated mitochondria illustrated in fig.1 shows that the effect sets in with a lag period of several minutes reaching its maximum after ~15 min. A similar relationship exists with respect to insulin in isolated fat cells [16]. This and the finding, that there is no additive effect if insulin and H_2O_2 are given together (not shown) suggests that the two agents may share a common mechanism in PDC activation.

In the studies shown in fig.1 we could further demonstrate that the effect of H_2O_2 is not specific, and that an organic peroxide, *t*-butyl hydroperoxide, is also effective in PDC activation displaying a similar time relationship. On the other hand comparison of the concentration relationships in fig.2 clearly shows that *t*-butyl hydroperoxide is much more effective than H_2O_2 leading to half-maximal increase in PDH_a activity already at ~5 μM. This difference may be attributable to a greater stability of the organic hydroperoxide which is known to react only with glutathione peroxidase [17], while H_2O_2 is decomposed by both glutathione peroxidase and catalase [17], the latter a likely contaminant of mitochondrial preparations. It should be noted that *t*-butanol, the product of *t*-butyl hydroperoxide reduction did not change PDC activities when added to mitochondria at comparable concentrations (not shown).

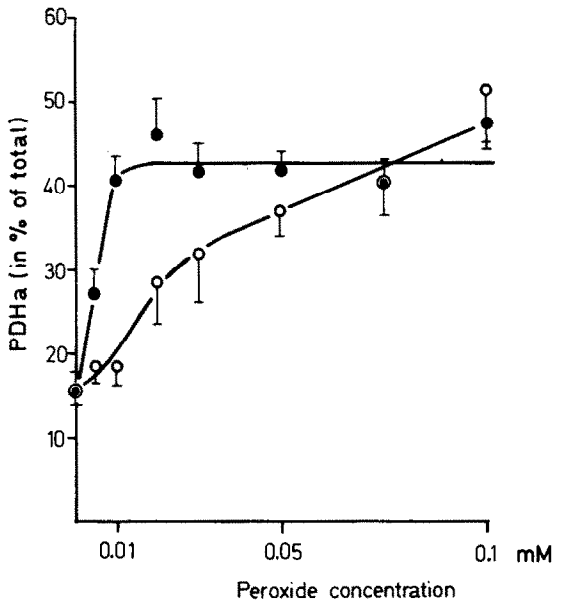

Fig.2. PDH_a activity of isolated fat cell mitochondria as a function of the concentration of H_2O_2 (○) and *t*-butyl hydroperoxide (●). Mitochondria were incubated in KCl-medium containing 0.05% BSA and the peroxide concentrations as indicated for 9 min at 25°C. Data points are the means ± SEM from 5 separate expt. With the exception of the lowest $[H_2O_2]$ all points differ significantly with $p < 0.05 - < 0.01$ from the controls. Total PDC activity (100%) was 12.4 ± 2.4 mU/assay. For further experimental details see section 2.

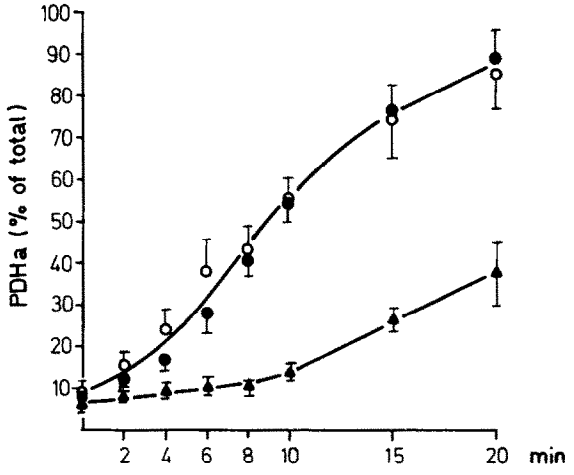

Fig.1. Time course of PDC activation by peroxides. Fat cell mitochondria corresponding to a total PDC activity of 9.8 ± 1.2 mU were incubated at 25°C in 1.5 ml KCl-medium containing 0.05% BSA for the times indicated in the abscissa. Each point represents the means of different experiments ± SEM as indicated: (●) 0.1 mM hydrogen peroxide (n = 8); (○) 0.1 mM *t*-butyl hydroperoxide (n = 3); (▲) controls (n = 5).

From a physiological point of view it seems of interest that insulin leads to increased H_2O_2 production in adipocytes as judged from [^{14}C]formate oxidation [7]. This is confirmed by experiments documented in table 3 where there was a 2-fold stimulation of formate oxidation by insulin accompanied by a 3-fold increase in PDH_a activity.

However in contrast to other reports [5,18] our data in table 3 demonstrate that the effect of insulin on both PDC activity and [^{14}C]formate oxidation was dependent on the addition of glucose to the

Table 3
Effect of insulin on PDC activity and formate oxidation in isolated fat cells

Experimental condition	Glucose added	PDH_a (in % of total)	[^{14}C]Formate oxidation (% of the basal rate)
Control	None	9.6 ± 1.9	
Insulin 2 mU/ml	None	10.1 ± 2.4	107 ± 10
Control	0.5 mM	12.2 ± 1.8	
Insulin 2 mU/ml	0.5 mM	36.1 ± 2.8	211 ± 25

Mean values ± SEM from 5 different expt., each assayed in triplicate are given. Cells corresponding to a total PDC activity of 23.2 ± 2.8 mU were incubated 30 min at 37°C in 2 ml 'Hepes buffer' containing 2.5% BSA, 0.2 mM [^{14}C]formate and glucose as indicated. For further details see section 2

incubations. However, PDC activation by exogenously added H_2O_2 (5 mM) did not depend on the presence of glucose in the medium. The mean values from 2 expt. for PDH_a activity (in % of total activity) in controls and H_2O_2-stimulated cells were 6.5 and 27.0 in the absence, and 5.3 and 30.1 in the presence of glucose (0.5 mM). It appears therefore that glucose has to serve as substrate for the insulin-stimulated intracellular peroxide generation and hence activation of the PDC system.

From these results, isolated fat cell mitochondria can be regarded as the preferred object for the study of the mechanism of PDC activation by peroxides. Concerning the signal transfer from the plasma membrane to the mitochondria the high efficiency of *t*-butyl hydroperoxide points to the possibility that not H_2O_2 itself but an intracellularly generated organic peroxide might be the postulated messenger of insulin. With respect to the mechanism of PDC interconversion it has been proposed that the kinase (which leads to inactivation) is activated by reduction (and acetylation) of the lipoyl moieties of the lipoate acetyltransferase [19,20]. Accordingly, an increase of the proportion of oxidized transferase-bound lipoyl residues would deactivate the kinase and thus lead to PDC activation. It seems therefore conceivable that the mechanism of peroxide(s) (and insulin) implies enzyme catalyzed lipoyl-SH oxidation possibly involving mitochondrial glutathione peroxidase or (an)other peroxidative enzymatic pathway(s).

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